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Fluorescence-activated sorting of totipotent embryonic stem cells expressing developmentally regulated *lacZ* fusion genes

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ABSTRACT **Murine embryonic stem (ES) cells were infected with a retrovirus promoter trap vector, and clones expressing *lacZ* fusion genes (LacZ$^+$) were isolated by fluorescence-activated cell sorting (FACS). Of 12 fusion genes tested, 1 was repressed when ES cells were allowed to differentiate *in vitro*. Two of three *lacZ* fusion genes tested were passed into the germ line, indicating that FACS does not significantly affect stem cell totipotency. The pattern of *lacZ* expression observed *in vivo* was consistent with that seen *in vitro*. Both fusion genes were expressed in preimplantation blastulas. However, a fusion gene whose expression was unaffected by *in vitro* differentiation was ubiquitously expressed in day-10 embryos, while the other, which showed regulated expression *in vitro*, was restricted to cells located along the posterior neural fold, the optic chiasm, and within the fourth ventricle. These results demonstrate the utility of using promoter trap vectors in conjunction with fluorescence sorting to disrupt developmentally regulated genes in mice.**

Mammalian development is a regulated process whereby the structural and functional organization of the animal is progressively expressed. Transplantation experiments (1, 2) and studies with morphogenic determinants (3) demonstrate that many developmental processes are programmed by extracellular signals acting in a temporally and spatially defined manner. Developmentally important genes have been isolated from flies and nematodes by analyzing mutant individuals (4, 5). Although mammalian genes important in development can be isolated, the process is relatively slow and is limited to a small number of mutant alleles (6).

An alternative approach has used embryonic stem (ES) cells to study gene functions in mice. ES cells can be genetically manipulated *in vitro* while maintaining the ability to colonize the germ line of a developing embryo. In this manner, ES cells provide a means to introduce genes and mutations into mammalian organisms (7, 8). These include genes disrupted by homologous recombination, thus allowing the organismal functions of cloned genes to be assessed.

While targeted mutations are extremely important, it would also be desirable to identify previously unknown genes affecting organismal phenotypes, since genes required for biological function (and disease) usually cannot be predicted in advance. To be useful, such approaches should (*i*) target most genes, (*ii*) permit efficient screening of mutagenized cells for mutations of interest, and (*iii*) allow for isolation of the mutated genes.

Several types of targeting vectors have been described that confer selectable phenotypes when cellular genes are disrupted (9–15). After transfer into the germ line, ≈40% of disrupted genes cause obvious phenotypes when bred to the homozygous state (14, 16). The process is extremely efficient, such that the number of mutant alleles exceeds what can be easily analyzed at an organismal level. Therefore, methods to select for particular types of mutations would be highly desirable. Toward this end, the *Escherichia coli lacZ* gene has been used to identify mutations in developmentally regulated genes (10, 14). Integration sites have also been sequenced to identify mutations affecting known genes (16).

In the present study, ES cells were infected with a retrovirus vector (U3LacZ) that induces *lacZ* gene fusions when cellular genes are disrupted as a result of virus integration (13). We demonstrate that the fluorescence-activated cell sorter (FACS) can be used to screen large pools of infected ES cells for mutant clones expressing *lacZ* fusion genes (LacZ$^+$). These clones were analyzed to identify genes that were repressed upon differentiation *in vitro* and the disrupted alleles were introduced into the germ line.

MATERIALS AND METHODS

Viruses and Cell Culture. ES-D3 (129; XY; agouti/agouti) cells (1.5×10^6 cells) were infected with the U3LacZ virus as described (13) at a multiplicity of infection (moi) of 1 neomycin-resistant (neo^R) colony-forming unit (cfu) per cell as titered on NIH 3T3 cells. The producer line ψ2(BG2)4 (13) was used as a source of virus (titer, 4×10^6 neo^R cfu per ml per 10^7 producer cells). ES cells were cultured on irradiated mouse embryo fibroblast feeder layers (prepared from day-13 embryos) in Dulbecco's minimum essential medium (DMEM) supplemented with 15% fetal calf serum (heat inactivated at 55°C for 30 min), 100 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 10 units of penicillin per ml, 10 μg of streptomycin per ml, and 1000 units of leukemia inhibitory factor per ml (Esgro; GIBCO).

Isolation of LacZ$^+$ ES Cell Clones by FACS. Cells (1×10^6) were stained with fluorescein di-β-D-galactopyranoside (FDG) in 0.1 ml of staining medium [phosphate-buffered saline (PBS)/10 mM Hepes, pH 7.3/4% fetal bovine serum] mixed with an equal volume of prewarmed (37°C) 2 mM FDG in water for 1 min. FDG loading was stopped by returning the cells to isotonic conditions by adding 0.5 ml of ice-cold staining medium with 1 μg of propidium iodide per ml. ES cells were sorted with a Becton Dickinson FACStar Plus cell sorter primarily as described (17). Data analysis was performed with Consort 40 software (Becton Dickinson).

Nucleic Acid Blot Hybridization and RNase Protection Assays. Nucleic acid blot hybridization using a *lacZ* probe [3-kilobase-pair (kbp) *Nhe* I/*Eco*RI fragment] from pGg-TKNeoU3LacZen(−) and RNase protection analysis were carried out as described (13).

***In Vitro* Differentiation of ES Cells.** ES cells were treated with trypsin and grown in suspension culture in DMEM supplemented with 10% fetal calf serum and 10 units of penicillin and 10 μg of streptomycin per ml. After 6 days, a mixture of simple and cystic embryoid bodies were plated on

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Abbreviations: ES cell, embryonic stem cell; FACS, fluorescence-activated cell sorting; moi, multiplicity of infection; FDG, fluorescein di-β-D-galactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-galactopyranoside; LTR, long terminal repeat; nt, nucleotide(s).

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gelatinized dishes and cultured for 4 days. Cells were assayed for *lacZ* expression by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining as described (13).

Construction of Chimeric Animals. Germ-line contribution of U3LacZ-infected ES-D3 cell lines expressing *lacZ* fusion genes was tested by injecting 15–20 ES cells into C57BL/6J blastocysts (3.5 days). Ten to 14 blastocysts were implanted per uterus in pseudopregnant ICR (Harland) mice (18). Chimeric male offspring were mated with C57BL/6J mice.

Embryonic Expression of *lacZ* Fusion Genes. Blastocysts (3.5 days) and 10-day postcoitum (p.c.) embryos were fixed in PBS/2% paraformaldehyde/0.2% glutaraldehyde for 10 min at 4°C and rinsed in PBS for 1 hr at 4°C before staining. Embryos were stained in PBS/0.02% Nonidet P-40/0.01% SDS/2 mM $MgCl_2$/5 mM $K_3Fe(CN)_6$/5 mM $K_4Fe(CN)_6$/1 mg of X-Gal per ml, pH 7.2. The embryos were stained overnight and washed in PBS.

RESULTS

Isolation of ES Cell Clones Expressing *lacZ* Fusion Genes. The U3LacZ retrovirus contains the coding sequence of the *E. coli* *lacZ* gene inserted into the U3 region of a replication-defective Moloney murine leukemia virus (13). *lacZ* sequences form part of the long terminal repeats (LTRs) that flank the provirus and the 5′ copy of *lacZ* is inserted just 30 bp from the flanking cellular DNA. The U3LacZ vector also encodes the *neo* gene under transcriptional control of the herpes simplex type 2 thymidine kinase (tk) promoter (19), which provides an independent measure of virus titers (see Fig. 2*A*). In NIH 3T3 cells, only 0.4% of all integrated proviruses expressed β-galactosidase as judged by X-Gal staining (13). Cell clones that expressed β-galactosidase contained proviruses in which the *lacZ* gene in the 5′ LTR was expressed on fusion transcripts initiating in the flanking cellular DNA. Sequences adjacent to expressed U3 genes have characteristics of 5′ regions of genes transcribed by RNA polymerase II. In particular, they hybridize to single-copy DNA and transcripts expressed in uninfected cells, and some contain functional promoters (16, 20). In one case, integration of the provirus disrupted a known gene (16). The number of sites in the genome that activate U3 gene expression appears to be comparable to the total number of expressed genes (11, 13).

ES cells were infected with the U3LacZ virus and $LacZ^+$ clones were isolated by two rounds of FACS–FDG (17). Infected and mock-infected ES cells were detached by treatment with trypsin 48 hr after infection, the fluorogenic substrate FDG was introduced by hypotonic shock, and the cells were sorted. Propidium iodide was added to exclude dead cells (which comprised <10% of all cells; data not shown). Background fluorescence was observed in mock-infected cells due to lysosomal β-galactosidase (21). However, while fluorescence resulting from the cytoplasmic *E. coli* enzyme occurs predominantly in the green (520–530 nm) wavelengths, the product of the lysosomal enzyme was detected in both the green and orange (560–580 nm) wavelengths (22). Consequently, enrichment of $LacZ^+$ ES cells was improved by sorting for green single-positive cells while excluding orange/green double-positive cells.

Fluorescence profiles of mock-infected and U3LacZ-infected ES cells prior to sorting are shown in Fig. 1 *A* and *D*, respectively. These profiles do not differ significantly since $LacZ^+$ cells represent only a small fraction of the infected cell population. Isolation of rare $LacZ^+$ cells was complicated by the relatively rapid diffusion of fluorescein out of $LacZ^+$ cells even when the cells were maintained at 4°C. A two-step strategy was therefore used to isolate ES cells expressing *lacZ* fusion genes. In an initial enrichment step, cells were sorted only by fluorescence intensity set to exclude 98% of mock-infected cells (23). This allowed rapid sorting (>10,000 cells per sec), thus minimizing problems with background, but only

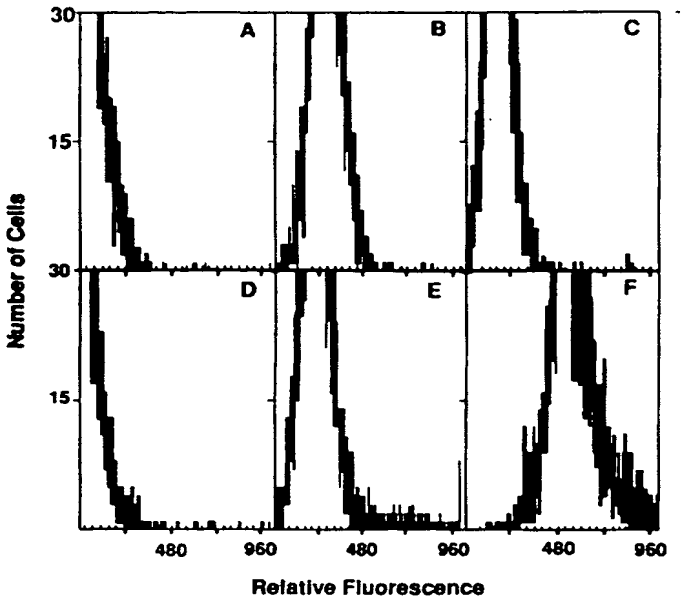

FIG. 1. Fluorescence profiles of mock-infected and U3LacZ-infected ES cells. Cells were stained with FDG and propidium iodide and fluorescence was analyzed by FACS 48 hr after mock infection (*A*) or after infection with the U3LacZ virus (*D*). Cells from mock-infected and U3LacZ-infected cultures, which displayed the highest fluorescence intensity (i.e., a level that excluded 98% of mock-infected cells), were re-sorted (*B* and *E*, respectively). Individual cells exhibiting the highest fluorescence levels from both mock-infected and U3LacZ-infected cell populations were grown in mass culture and were analyzed (*C* and *F*, respectively).

resulted in a 20- to 30-fold enrichment (Fig. 1 *B* and *E*). In a second step, cells obtained during the first round of sorting were expanded in mass culture and sorted by both forward scatter and fluorescence intensity. Individual cells exhibiting high fluorescence intensity (when compared to mock-infected cells) were sorted into 96-well dishes. After 5–6 days, 20–30% of the wells contained single, undifferentiated colonies, a number similar to the plating efficiency of unsorted ES cells. This two-step procedure resulted in a significant enrichment of $LacZ^+$ cells (Fig. 1 *D–F*); >95% of the clones obtained after two rounds of sorting expressed β-galactosidase as judged by X-Gal staining. $LacZ^+$ ES cells were recovered only from cultures infected with the U3LacZ provirus, whereas cells with higher levels of endogenous β-galactosidase were not recovered from mock-infected cultures even after two rounds of sorting (Fig. 1 *A–C*).

Sixteen $LacZ^+$ clones were isolated from separate infections to ensure that each was clonally independent and analyzed by Southern blot hybridization. Of these, 75%, 18%, and 5% contained one, two, and three proviruses, respectively (data not shown), consistent with the moi of 1 (as estimated by titering the virus on NIH 3T3 cells). This suggests that ES cells and NIH 3T3 cells are equally susceptible to infection by retroviruses. Approximately 30% of the proviruses were rearranged as judged by Southern blot analysis. In the three clones examined further, provirus sequences appeared to have been deleted as a result of homologous recombination between the elongated LTRs (data not shown). This is a much higher incidence of deletions than was observed in NIH 3T3 cells (13) and may reflect selection against a transcriptional silencer located in the tRNA primer binding site (24–26).

$LacZ^+$ Expression Is Activated by Flanking Cellular Promoters. To determine whether transcripts in $LacZ^+$ clones initiate within the flanking cellular DNA, total RNA was

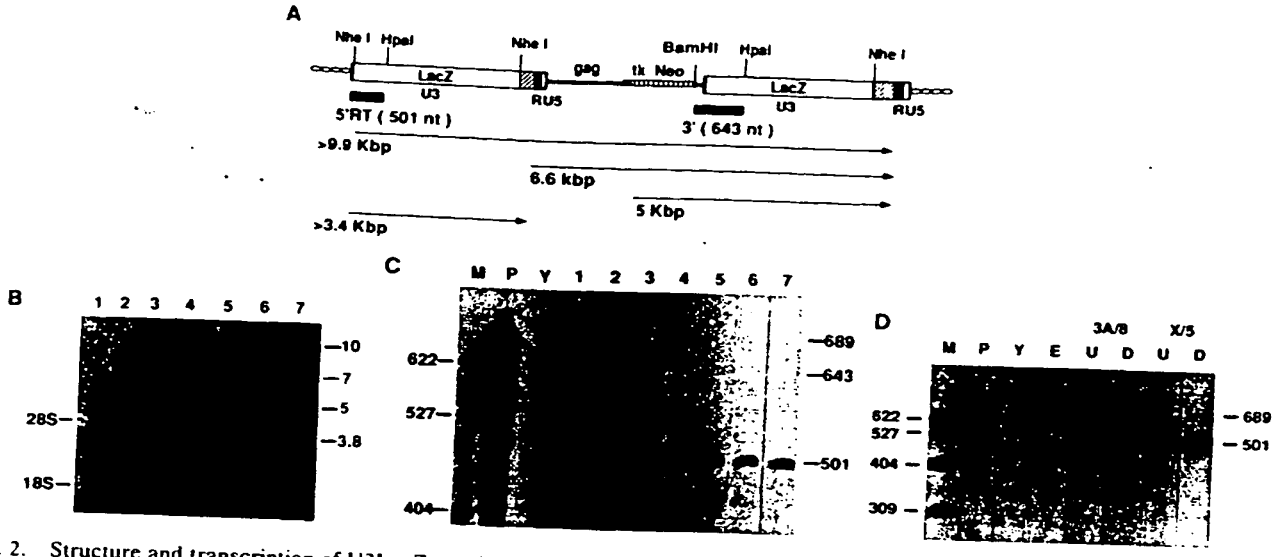

FIG. 2. Structure and transcription of U3LacZ provirus. (*A*) In NIH 3T3 cells, 5- and 6.6-kb transcripts initiate at the internal tk and LTR promoters, respectively, and extend through the right-hand LTR (13). Cell–*lacZ* fusion transcripts initiating various distances upstream in the flanking DNA and terminating at either the 5′ or 3′ LTR are expressed only in clones expressing β-galactosidase. Transcripts extending through the 5′ and 3′ LTRs protect 501 and 643 nt of a probe spanning the region between the *Bam*HI and *Hpa* I sites, respectively. (*B*) Northern blot analysis of provirus transcripts. Ten micrograms of cellular RNA was fractionated on 1% formaldehyde/agarose gels, transferred to nitrocellulose, and hybridized to *lacZ* probes as follows: lane 1, uninfected ES cells; lane 2, LacZ⁻ (white) NIH 3T3 clone (WIC) containing U3LacZ provirus (13); lane 3, LacZ⁺ (blue) NIH 3T3 clone (CD15) containing U3LacZ provirus (13); lanes 4–7, LacZ⁺ ES cell clones 9/12, 3A/8, X/5, and 2/3/9. Sizes (kb) of the expected transcripts are indicated on the right. (*C* and *D*) RNase protection analysis of provirus transcripts. (*C*) RNAs analyzed: lane P, undigested probe; lane M, size markers (nt); lane Y, yeast tRNA; lanes 1–7, same as in *B*. (*D*) RNAs analyzed: lane Y, yeast tRNA; lane E, D3 RNA; lanes U and D, RNA from undifferentiated and differentiated 3A/8 and X/5 LacZ⁺ clones, respectively; lane M, end-labeled DNA molecular size markers; lane P, undigested probe. Positions of size markers (left) and mobilities of the expected fragments (right) are indicated in nt.

analyzed by an RNase protection assay (13). RNA from LacZ⁺ ES cell clones invariably protected a 501-nucleotide (nt) fragment, as expected for hybrid cell–virus transcripts initiating in the flanking cellular DNA and extending through the 5′ end of the LTR. Unlike NIH 3T3 cells, transcripts extending through the 3′ LTR, which protect a 643-nt frag-

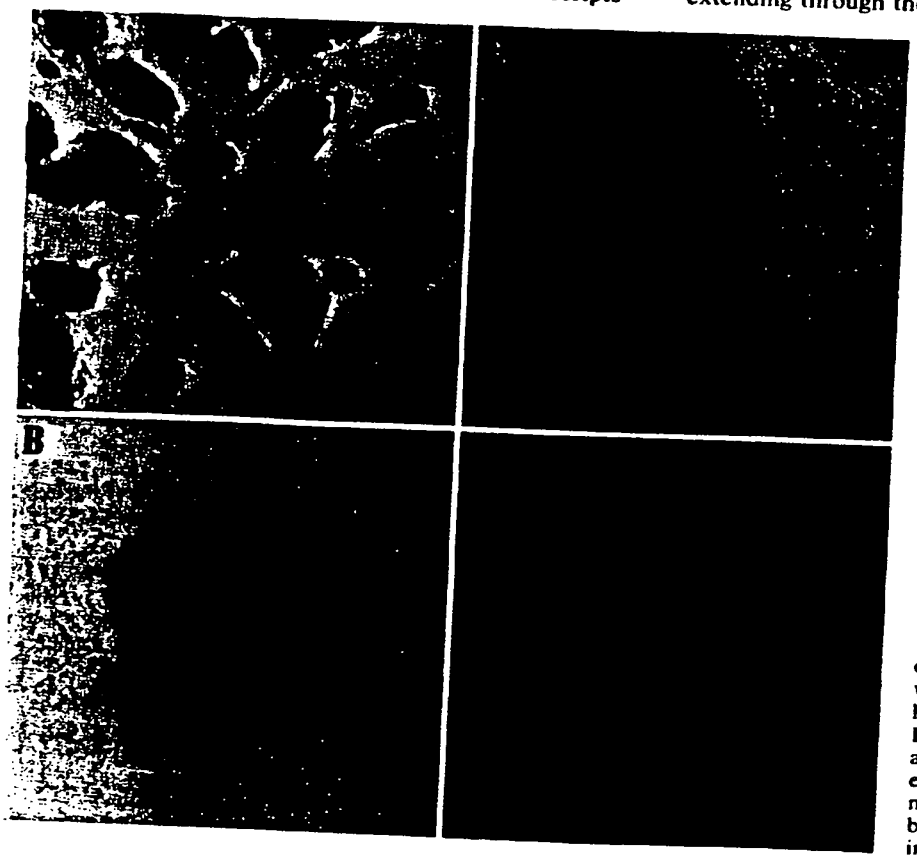

FIG. 3. Differential expression of *lacZ* fusion genes *in vitro*. Cells were stained with X-Gal as follows: *A* and *C*, undifferentiated ES cell colonies from clones 3A/8 and X/5, respectively; *B*, 3A/8 embryoid body showing heterogeneous staining; *D*, X/5 embryoid body showing homogeneous staining.

ment, were only present at low levels since the Moloney murine leukemia virus and the *tk* promoters are transcriptionally inactive in ES cells (27–29) (Fig. 2*C*). This accounts for the fact that only $1/10^6$ cells infected with the U3LacZ virus at a moi of 1 acquired *neo* resistance. The rare *neo*-resistant clones probably reflect instances in which the proviruses contain deletions or have integrated near strong cellular promoters and in regions that allow proviral sequences to be removed by RNA splicing (30, 31). Finally, as judged by Northern analysis, only 100–500 nt of cellular RNA were fused to *lacZ* transcripts (Fig. 2*B*), indicating that U3LacZ expression results from integrations in or near the 5′ exons of expressed genes.

***In Vitro* Differentiation of LacZ$^+$ ES Cell Clones.** Under appropriate culture conditions ES cells form aggregates of differentiated cells known as embryoid bodies. Simple embryoid bodies consist of ES cells surrounded by a layer of endodermal cells, while cystic embryoid bodies develop an additional layer of columnar ectoderm-like cells around a fluid-filled cavity. When allowed to reattach onto tissue culture surfaces, both simple and cystic embryoid bodies differentiate into a variety of cell types (32). To determine whether any of the *lacZ* fusion genes were regulated during differentiation, 12 LacZ$^+$ clones were allowed to form embryoid bodies and were stained with X-Gal. In 1 (clone 3A/8) the *lacZ* fusion gene was repressed upon differentiation as judged by X-Gal staining (Fig. 3 *A* and *B*). Although residual β-galactosidase activity was detected by X-Gal staining, RNA from 3A/8 embryoid bodies failed to protect the 501-nt fragment of the cell–*lacZ* fusion transcript (Fig. 2*D*). Fusion transcripts were also not detected by Northern blot hybridization; however, β-actin gene expression was unaffected (data not shown). This suggests that the fusion gene was repressed in a majority of cells present in the embryoid bodies. It is interesting to note that transcripts initiating from the 5′ LTR and *tk* promoters were absent even in differentiated ES cells since differentiation has been shown to activate expression of the LTR (33).

Germ-Line Transmission of Cell–*lacZ* Fusion Genes. To test whether LacZ$^+$ ES cells isolated by FACS maintained totipotency, C57BL6 blastocysts were injected with cells derived from three LacZ$^+$ clones. Two clones, 3A/8 and X/5, produced male chimeras with a high degree (50–90%) of coat color chimerism. One X/5 and four 3A/8 chimeric males have currently transmitted the D3 agouti coat color marker to their progeny; 15% of the progeny of the X/5 founder and 15–100% of the progeny of the 3A/8 founder animals were agouti. These results demonstrate that selection for U3LacZ expression by FACS–FDG did not prevent D3 cells from contributing to the male germ line.

***In Vivo* Expression of *lacZ* Fusion Genes.** *lacZ* expression patterns *in vivo* were analyzed in blastocysts (3.5 days) and day-10 p.c. embryos obtained from mating 3A/8 and X/5 transgenic animals heterozygous for the U3LacZ provirus with C57BL/6J mice. The inner cell mass of approximately half of the blastocysts derived from such crosses stained with X-Gal [Fig. 4 *A* (*Left*) and *C* (*Right*)]. *lacZ* expression within the inner cell mass is noteworthy since ES cells are derived from comparable cells recovered from blastocysts after implantation is delayed (32). Furthermore, by day-10 p.c., X/5 embryos stained homogeneously with X-Gal (Fig. 4*D Right*), whereas 3A/8 embryos showed a restricted pattern of *lacZ* expression (Fig. 4*B Left*). In particular, staining was localized in two areas where tissue morphogenesis or reorganization was taking place: (*i*) the ependymal and mantle layers of the neural tube starting midway between the forelimb and hindlimb bud and extending to the tail, and (*ii*) the optic chiasm (Fig. 4*B Left*). Staining was also seen in the wall of the fourth ventricle medial to the auditory vesicle. Thus, consistent with regulation of *lacZ* activity *in vitro*, the *lacZ* fusion gene was transcriptionally active in X/5 embryos and repressed in most tissues of 3A/8 embryos.

DISCUSSION

DNA- and retrovirus-mediated gene transfer has previously been used as a means of insertional mutagenesis in preimplantation embryos (34–40) and in ES cells (9, 10, 14–16). As with vectors that rely on splicing to generate fusion transcripts, the U3LacZ promoter trap vector offers several advantages for disrupting genes in totipotent ES cells. First, studies with a splice trap vector (14) and promoter trap vectors (16) have shown that 9/24 and 2/4 integrations result in embryonic death when bred to homozygosity. Therefore, selection for cells containing fusion genes significantly reduces the number of recombinants needed to screen for loss of gene function, as compared to cells in which viruses have integrated randomly.

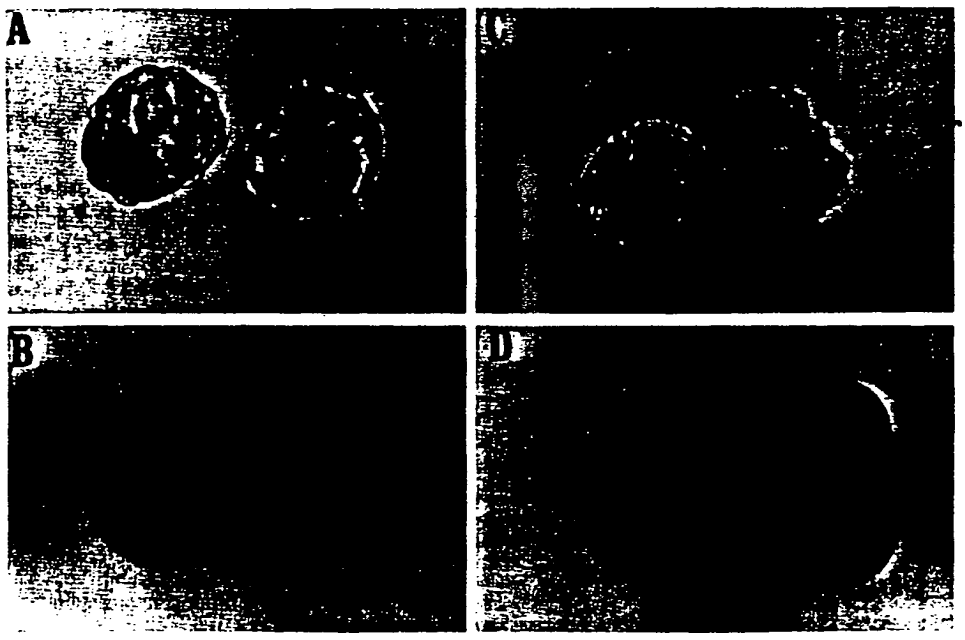

FIG. 4. Differential expression of *lacZ* fusion genes *in vivo*. Embryos were isolated after 3.5 and 10 days p.c. and stained with X-Gal as follows: *A* and *C*, 3.5-day p.c. 3A/8 and X/5 embryos, respectively; *B* and *D*, 10-day p.c. 3A/8 and X/5 embryos, respectively. Control embryos, which did not stain, are located on the right of each pair in *A* and *B* and on the left of each pair in *C* and *D*.

Second, as retrovirus integration, unlike DNA-mediated gene transfer (41), preserves both viral and cellular sequences, cloning of cellular genes responsible for recessive phenotypes is greatly simplified. Furthermore, as selection for β-galactosidase favors integrations in or near 5′ exons, isolation of cDNA clones of genes disrupted by the U3LacZ provirus is simplified as compared to vectors in which expression of a marker gene is activated by transcriptional enhancers (9, 10, 14). Third, the number of sites in the genome that can activate U3 gene expression ($2–4 \times 10^4$) is similar to the total number of expressed genes, as judged by RNA renaturation kinetics (11, 13, 42). Since the displaced genes are functionally inactivated (W. Chang, C. Hubbard, C. Friedel, and H.E.R., unpublished data), it appears that most expressed genes can be targeted by promoter trap selection. Finally, as β-galactosidase can be visualized at the cellular level and is not toxic to mammalian cells, mice expressing U3LacZ fusion genes can be used to study developmental patterns of gene expression *in vivo* (9, 10, 14, 43).

This strategy offers several advantages over cDNA subtraction as a means to identify regulated genes. First, the process is sensitive and not biased for highly expressed genes. This is important since many genes are expressed at levels too low for efficient recovery after cDNA subtraction. Second, the vectors insert in or near 5′ exons (11, 13), sequences that are often missing from all but full-length cDNA clones. Third, differential expression of the U3 gene is probably related to changes in promoter activity rather than mRNA stability, transport, or processing, although appended cellular RNA could occasionally confer posttranscriptional regulation.

Finally, FACS–FDG does not affect stem cell totipotency. As up to 10^7 cells can be sorted in an hour, the ability to sort totipotent ES cells should allow large numbers of cells containing independent proviral integrations to be rapidly sorted into $LacZ^+$ and $LacZ^-$ populations. Such sorting techniques will facilitate the generation of both expressed ($LacZ^+$) and null ($LacZ^-$) ES cell libraries in which the U3LacZ proviruses have integrated next to transcriptionally active and inactive sites, respectively. These libraries can then be used to select for clones in which expression of *lacZ* fusion genes is altered by morphogens or differentiation. Multicolor sorting with FDG and other cellular markers may also increase the efficiency with which mutant ES cells can be recovered.

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